supporting material surface via its connection to the ion bearing functional group; and

said hydrophobic functional group forming a permeable stationary layer shielding said ion bearing functional group from full contact with the mobile phase.

REMARKS

This Amendment responds to the Office Action Dated 11/30/04, which had a one-month response time; meaning that the Amendment is being submitted within the two month extension period window. Please use the accompanying PTO-2038 form as authorization to pay the $225 fee for this extension, for the small entity inventors. Please also enter into this application the submitted Power of Attorney to the undersigned for prosecuting the application.

Original Claims 1-7 in the application are being cancelled and new Claim 8 is being submitted with this Amendment.

The status of claims in the application now thus is:
Claims 1-7, originally filed but now cancelled; and
Claim 8, newly added with this amendment.

The Office Action required an election of species of different functional groups, identified as sulfate, phosphate, carboxylic acid, amine, secondary amine and tertiary amine. However, These identified chemical groups were mentioned only in the depending claims, which have now been cancelled. New Claim 8, the only claim now in the application, makes no mention of any of these chemical groups. Instead, the invention of Claim 8 lies in the recited association of

the ion bearing and hydrophobic functional groups to each other and to the rigid supporting material and mobile phase.

Accordingly, the undersigned herein respectfully traverses any need to make a selection of any of the identified "species", because none is recited in the pending Claim 8.

Of related interest, the noted chemicals (sulfate, phosphate, carboxylic acid) are commonly used to provide an acidic ion bearing functional group suited to yield an cation exchange, while the noted chemicals (amine, secondary amine, tertiary amine) are commonly used to provide a basic ion bearing functional group suited to yield an anion exchange. Even other chemicals or combinations can be used in the stationary phase for liquid chromatography analysis, and their specific identification really forms no part of or restriction to the invention as now claimed in Claim 8.

The publications cited by reference numbers on page one of the specification are being identified on the accompanying sheet and copies of each are also being submitted herewith, as requested.

Favorable consideration of the application as now presented is requested. The undersigned would welcome the examiner's call if such could help in the examination and prosecution of the application.

Respectfully submitted,

Charles F. Lind

Charles F. Lind
PTO Registration No. 20,155
PTO Customer No. 000045580

Application No. 10/627,636; Filed 07/28/04
Art Unit 1723; Examiner Ernest G. Therkorn
For: UNIVERSAL BONDED PHASE MATERIALS FOR CHROMATOGRAPHIC SEPARATION
Attorney Docket CFL 27613

References cited by footnote numbers on page 1 of application specification:

[1] Snyder, L. B. et; Introduction to Modern Liquid Chromatography, second Edition, JW&S (1979), page 285.

[2] Boppana, Venkata K. et al; Journal of Chromatography, 631 (1993), page 251.

[3] O'Gara, John E. et al; LCGC North America (2001), 19(6), page 632.

[4] Przybyciel, Matthew et al; LCGC North America (2002), 20(6), page 516.

Ref # 1

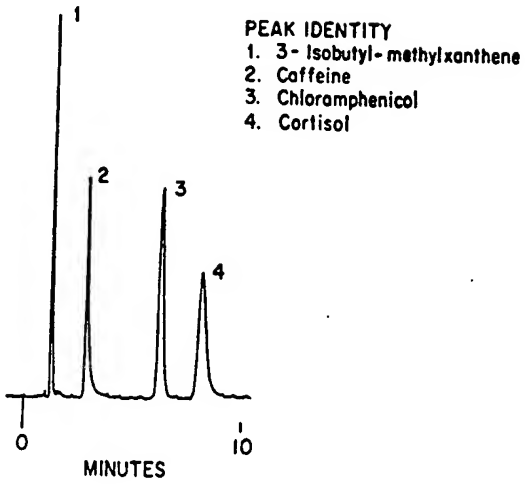

Figure 7.7 Normal-phase separation of drug mixture with polar bonded-phase column. Column, 25 × 0.46 cm, Zorbax-CN; mobile phase, 15%v isopropanol in hexane; pressure, 750 psi; temp., ambient; detector, UV, 254 nm, 0.32 AUFS. Reprinted from (*25*) with permission.

convenient and effective polar solvents for the selectivity extremes. If the right solvent selectivity is not obtained with mixtures of one of these solvents plus, for example, hexane, then ternary mobile phases based on *two* of these "extreme-selectivity" solvents plus hexane may be of help (see example of Figure 11.4). If this approach is not successful, then some other variable (e.g., temperature) will probably be required to improve selectivity and band spacing. An example of a normal-phase BPC separation is shown in Figure 7.7. In this case a "nitrile" bonded-phase packing was used as the stationary phase and hexane modified with isopropanol was the mobile phase. To elute very polar solutes, stronger base-solvents (e.g., butyl chloride or dichloromethane instead of hexane) can be used with polar modifiers such as methanol.

Mobile Phases in Reverse-Phase BPC. The dependence of solvent strength and selectivity in reverse-phase BPC is similar to the case of reverse-phase LLC (Section 8.4). Usually water is used as base solvent, to which varying concentrations of miscible organics are added. Table 7.4 summarizes the relative strengths (less polar solvents with smaller P' values are stronger) of some solvents that have been used in reverse-phase BPC. Solvent strength is usually adjusted by varying the composition of the solvent mixture, and the data of Table 7.4 provide information on how k' changes with change in solvent composition. Methanol is the most commonly used organic solvent, since it meets the various requirements for a BPC solvent and is relatively cheap. Acetonitrile and tetrahydrofuran are the next most commonly used solvents, in that order.

Ref #2

Journal of Chromatography, 631 (1993) 251–254
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2619

Normal-phase high-performance liquid chromatographic determination of epristeride, a prostatic steroid 5α-reductase enzyme inhibitor, in human plasma

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ABSTRACT

An highly sensitive and selective high-performance liquid chromatographic method was developed for the determination of epristeride [17 β-(N-*tert.*-butyl carboxamido)-androst-3,5-diene-3-carboxylic acid, SK&F 105657], a potent inhibitor of the prostatic steroid 5α-reductase enzyme, in human plasma samples. Epristeride is currently in development for the treatment of benign prostatic hyperplasia. The analytical method involves isolation of epristeride and the internal standard [17 β-(N,N-diisopropyl carboxamido) estra-1,3,5 (10)-triene-3-carboxylic acid, SK&F 105419] from plasma by solid-phase extraction prior to chromatographic separation on an aminopropyl silica column, using hexane–methylene chloride–2-propanol–acetic acid as the mobile phase, with subsequent ultraviolet absorption detection. The absolute recovery of epristeride from plasma was 90.2 ± 2.9%. The limit of quantification for epristeride was 2.5 ng/ml. Linear response was observed for concentrations of epristeride ranging from 1 to 500 ng/ml plasma. The assay was sufficiently sensitive, accurate and precise to support pharmacokinetic studies in human subjects.

INTRODUCTION

Epristeride (17 β-(N-*tert.*-butylcarboxamido)-androst-3,5-diene-3-carboxylic acid, SK&F 105657, Fig. 1) is a potent and selective inhibitor of the prostatic steroid 5α-reductase enzyme, which converts testosterone to dihydrotestosterone (DHT) [1–3]. This steroid analogue is currently under development for the treatment of benign prostatic hyperplasia (BPH).

This report describes a sensitive and specific HPLC method for the determination of epristeride in human plasma samples. The approach involves isolation of the steroid analogue from plasma by solid-phase extraction followed by quantitative normal-phase chromatographic analysis with ultraviolet absorbance (UV) detection.

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MATERIALS AND METHODS

Chemicals

Epristeride (free acid, SK&F 105657, Fig. 1) and the internal standard (17 β-(N,N-diisopropyl carboxamido) estra-1,3,5(10)-triene-3-carboxylic acid, SK&F 105419, free acid, I.S.), were supplied by Drug Substances and Products, SmithKline Beecham Pharmaceuticals (Swedeland, PA, USA). Glacial acetic acid was obtained from Mallinckrodt (Paris, KY, USA). Monobasic potassium phosphate, dibasic potassium phosphate, HPLC-grade methanol, hexane, 2-propanol and methylene chloride were obtained from J. T. Baker (Phillipsburg, NJ, USA). Octadecylsilica (C_{18}) solid-phase extraction cartridges (100 mg, 1 ml) and the Vac-Elut manifold were purchased from Analytichem International (Harbor City, CA, USA).

Standard solutions and reagents

The stock standard solutions of epristeride and

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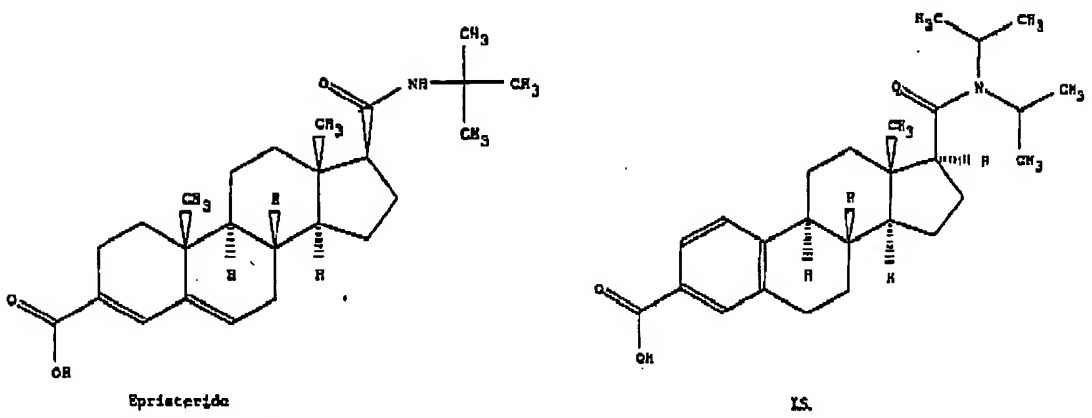

Fig. 1. Structures of epristeride and I.S.

the internal standard were prepared by dissolving 10 mg of the free acid in methanol in a 10-ml volumetric flask to give a final solution concentration of 1 mg/ml. Appropriate dilutions of the stock solution of epristeride were made with 50% aqueous methanol to generate a series of working standard solutions (100, 10, 1 and 0.1 μg/ml). All stock and working standard solutions were stable for 4 months when stored at 4°C. The stock solution of I.S. was diluted 1:50 with 50% aqueous methanol solution to give a solution concentration of 20 μg/ml. The solution was stable for 2 months when stored at 4°C.

Calibration

A set of plasma calibration standards (concentrations of epristeride; 0, 1, 2, 5, 10, 20, 50, 100, 200, 500 ng/ml) was analyzed with every determination of epristeride in plasma samples of unknown concentration, by adding appropriate volumes of the working standards of epristeride to 1.0 ml of heparinized plasma. The standard curve was established by weighted linear least-squares regression [weighting factor = $1/y$; due to the large difference between low and high concentrations of the analyte, the peak-height ratios (y) were weighted in order to get better estimates of analyte (x) concentrations] of the measured peak-height ratios of epristeride to internal standard *versus* the concentration of epristeride added to the plasma. Epristeride concentrations in unknown plasma samples were calculated from the following regression equation:

$$\text{Concentration of epristeride } (x) = (y-a)/b$$

where, b = slope of the regression line, a = y-intercept of regression line and y = peak-height ratio of epristeride to internal standard. The linear regression analysis was performed with a FIT FUNTION program of RS/1 software (version 4.3.1, BBN Research Systems, Cambridge, MA, USA). In FIT FUNCTION, the fitting is done in a series of iterations, in which the parameters are systematically adjusted by the Marquardt–Levenberg method until a least-squares solution is reached. The FIT FUNCTION program, besides generating The Equation Of The Best Fit, also compute Goodness-Of-Fit Statistics and generate ANOVA table calculating correlation coefficient (r^2) and standard deviation of the regression and also calculates the 95% confidence intervals for the standard curve.

Mobile phase

Hexane–methylene chloride–2-propanol–glacial acetic acid (410:50:40:1, v/v) is used as mobile phase.

Extraction of epristeride from plasma

The C_{18} extraction column was conditioned by successive washings with 1 ml of methanol and 1 ml of water. An aliquot of plasma (1 ml) containing 50 μl of 50% aqueous methanol (contains standards when preparing standard curve), 50 μl of internal standard solution (20 μg/ml, I.S.) and 500 μl of 0.5 *M* phosphate buffer, pH 8.0, were mixed in a 75 ×

12 mm borosilicate tube. The sample was then poured onto the C_{18} column and vacuum was applied. The column was washed successively with 3 ml water, 1 ml of 20% (v/v) aqueous methanol and 1 ml of hexane. The column was then eluted with 1 ml of mobile phase eluent and the eluate was collected into a 75 × 12 mm borosilicate tube. The eluate was evaporated under a gentle stream of nitrogen at 40°C and the residue was reconstituted in 200 μl of mobile phase and transferred to an autosampler vial. 10–75 μl were injected into the HPLC system for analysis.

High-performance liquid chromatography

The isocratic HPLC system consisted of a Beckman pump (Model 116, Beckman, Palo Alto, CA, USA) and an ultraviolet absorbance detector (Model 783, ABI, Ramsey, NJ, USA). Chromatographic separations were carried out on a 22 cm × 2.1 mm I.D. amino-propyl silica column (Pierce, Rockford, IL, USA) connected in-line to a 3 cm × 2.1 mm I.D. amino-propyl silica guard column. The column was maintained at room temperature and the mobile phase eluent, hexane–methylene chloride–2-propanol–glacial acetic acid (409:50:40:1, v/v), was pumped at a flow-rate of 300 μl/min. The mobile phase was filtered through a 0.2-μm nylon-66 filter and degassed before use. UV detection was accomplished at 274 nm. Samples were injected using an HPLC autosampler (WISP model 710B, Waters, Milford, MA, USA). The chromatographic data were collected with an automated laboratory system (Nelson, Cupertino, CA, USA).

Validation procedures

Four pools of plasma precision samples containing 2.5, 25, 250 and 450 ng/ml of epristeride were prepared by adding appropriate volumes of standard solutions to drug-free heparinized plasma. These plasma samples were stored at −20°C until analysis was performed. Five replicate samples from each pool were extracted and analyzed on three separate days. Concentrations were determined by comparison with a calibration curve prepared on the day of analysis. From the data obtained, intra-day precision (determined as the mean of the daily relative standard derivations, R.S.D.s), inter-day precision (determined as the R.S.D. of the daily means) and mean accuracy were calculated.

RESULTS AND DISCUSSION

The assay described here for the analysis of epristeride involves solid-phase extraction of the steroid from plasma as a preliminary isolation step, followed by quantitative HPLC analysis with subsequent UV absorbance detection. The high extinction coefficient of the molecule (exceeding 20 000 at 274 nm) and the use of HPLC columns of reduced internal diameter (2 mm) provided enhanced sensitivity for epristeride in developing highly sensitive analytical methodology for this molecule. The specificity of the method was also attributable to reversed-phase solid-phase extraction of the steroid from plasma samples followed by normal-phase chromatographic analysis of the plasma extract on a amino-propyl silica column, using hexane–methylene chloride–2-propanol–acetic acid as the mobile phase. Use of four component mobile phase is necessary to resolve the analyte and internal standard peaks from the endogenous plasma peaks and to obtain sharper peaks.

Recovery and stability

The recovery of epristeride and the internal standard from plasma was estimated with five determinations by comparing the peak height obtained with processed samples to that obtained by direct injection of an amount of standard equivalent to 100% recovery. At 25 ng/ml, a mean plasma recovery of 90.2 ± 2.96% was obtained for epristeride. In addition, epristeride and internal standard were found to be stable in the final extract at room temperature for at least 48 h.

Sensitivity, selectivity and linearity

By utilizing a 2.1 mm I.D. HPLC column, the on-column limit of detection of epristeride (signal-to-noise ratio 3) was 100 pg. Under the conditions used in this assay, the lowest concentration of epristeride that could be determined quantitatively in 1 ml of plasma samples was 2.5 ng. Calibration curves obtained were linear over the range of 1–500 ng/ml of epristeride. In this range, no interference either from endogenous substances or from the known metabolites of epristeride were observed. Weighted ($1/y$) linear regression analysis of calibration curves provided the equation $y = 0.03635\,x - 0.000307$ and a correlation coefficient greater than

TABLE I

ACCURACY AND PRECISION DATA FOR EPRISTERIDE IN HUMAN PLASMA

Parameter	Concentrations in plasma (ng/ml)			
	2.5	25	250	450
R.S.D. (%)				
Day 1	6.0	1.8	1.7	3.3
Day 2	4.8	6.3	3.4	5.3
Day 3	8.2	1.0	1.1	3.9
Error (%)[a]				
Day 1	−14.0	−8.1	−0.2	−3.0
Day 2	−18.8	−7.5	−2.4	−1.9
Day 3	−11.2	−14.9	−6.0	−5.8
Inter-day R.S.D.[b]	4.5	4.4	3.0	2.1
Intra-day R.S.D.[c]	6.3	3.0	2.0	4.2
Mean accuracy (%)	85.3	89.5	97.2	96.4

[a] (Calculated concentration − actual concentration)/actual concentration × 100.
[b] Coefficients of variation of daily means.
[c] Mean of the daily R.S.D.s.

0.999. The calibration curves were highly reproducible and the precision, as measured by the R.S.D.s at each of the spiked concentrations, was within 17% across the calibration range. The accuracy, evaluated by the average concentration back-calculated from the composite standard curve, was within 10% of the seeded value at each concentration.

Accuracy and precision

Table I summarizes the results obtained from a three-day validation study in which five replicate-seeded standards at four concentrations, 2.5, 25, 250 and 450 ng/ml, were analyzed each day by this methodology. The mean accuracy of the assay at these concentrations ranged from 85.3 to 97.2%, whereas the intra-day precision, indicated by the mean of the daily R.S.D.s, varied from 2.0 to 6.3%. The reproducibility of the assay was high with inter-day precision, indicated by the R.S.D.s of the daily means, ranging from 2.1 to 4.4%. Similar accuracy and precision results were obtained when the assay was revalidated with smaller volume of plasma (200 μl), but with a proportional drop in sensitivity.

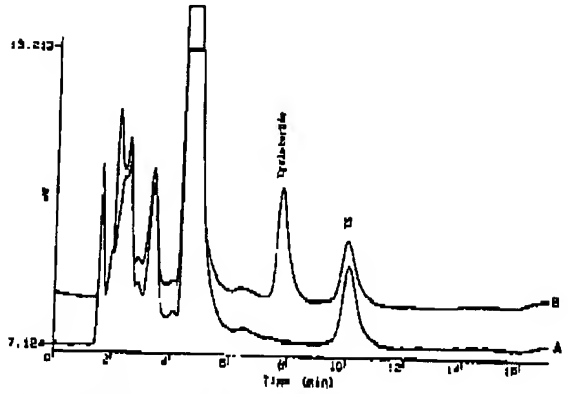

Fig. 2. Chromatograms of plasma extracts from blank human plasma with (A) I.S. and (B) plasma sample spiked with 20 ng/ml of eristeride.

Application of the procedure to plasma samples

The quantitative HPLC methodology described here provided for selective and sensitive detection of eristeride in human plasma samples. A typical chromatogram of a plasma extract obtained from drug-free plasma and a plasma sample spiked with 20 ng/ml of eristeride is shown in Fig. 2. The chromatography was highly reproducible and provided a retention time for eristeride and the internal standard of 7.9 and 10.2 min, respectively. To date the method has been used successfully in the analysis of biological samples from clinical studies. This method was also adopted for monkey, dog, rat and mouse plasma samples utilizing smaller volumes (100 μl) of plasma for quantification of eristeride in various pre-clinical pharmacokinetic studies.

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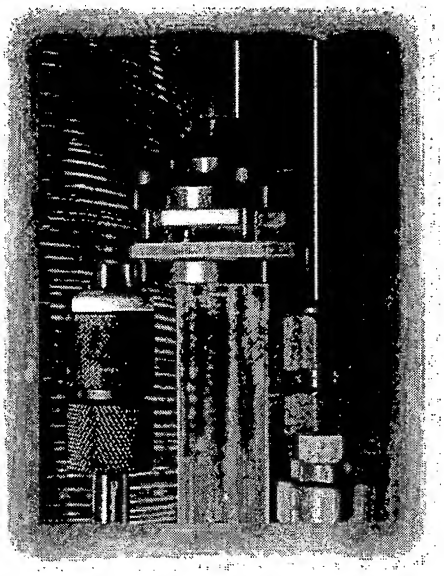
1 M. A. Levy, M. Brandt, D. A. Holt and B. W. Metcalf, *J. Steroid Biochem.*, 34 (1989) 571.
2 M. A. Levy, M. Brandt, J. R. Heys, D. A. Holt and B. W. Metcalf, *Biochemistry*, 29 (1990) 2815.
3 J. C. Lamb, H. English, P. L. Levandoski, G. R. Rhodes, R. K. Johnson and J. T. Isaacs, *Endocrinology*, 130 (1992) 685.

Ref. # 3

Embedded-Polar-Group Bonded Phases for High Performance Liquid Chromatography



In the past 10 years, embedded-polar-group bonded phases have become more popular with chromatographers. The characteristics of these phases are significantly different from traditional alkyl bonded phases, both with respect to chemical composition and chromatographic attributes. In this article, the authors chromatographically compare embedded-polar-group bonded phases with their purely alkyl counterparts. The authors describe first-generation, two-step and second-generation, one-step synthesis methodologies, and they report comparative retention mechanism studies for polar C8 and alkyl C8 phases. Embedded-polar-group phases differentiate themselves from their purely alkyl counterparts in three ways: improved peak shape for basic analytes, different selectivity, and stable retention in highly aqueous mobile phases.

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The properties of reversed-phase high performance liquid chromatography (HPLC) column stationary phases have changed significantly since the inception of this analytical technique more than 30 years ago. The chemical and physical properties of early column packings bear little resemblance to those found in current state-of-the-art materials. The primarily silica-based packings have gone from irregular to spherical in shape, from 10 µm to as small as 1 µm in diameter, and from low to high purity with respect to metallic contaminants. These material changes have led to dramatic improvements in column efficiency, stability, reproducibility, and basic analyte peak shape. At the same time, bonded-phase chemistries have remained virtually unchanged. Although column developers have achieved improved surface densities, octadecyl (C18) and octyl (C8) phases generally are made from the same silanes used 30 years ago.

In the early 1990s, conventional C18 and C8 bonded phases continued to yield large tailing factors for basic analytes (1). At that time, Nomura and co-workers (2) developed a novel type of bonded-phase methodology in which aminopropyl bonded phases were acylated to form an embedded polar amide functional group in the ligand's alkyl chain (see Figure 1). For example, Buszewski and colleagues (3) reported the reaction of aminopropyl silica with acid chlorides, phenylsulfonyl chloride, and alkyl isocyanates. These and subsequent bonded phases had reduced silanol interactions with

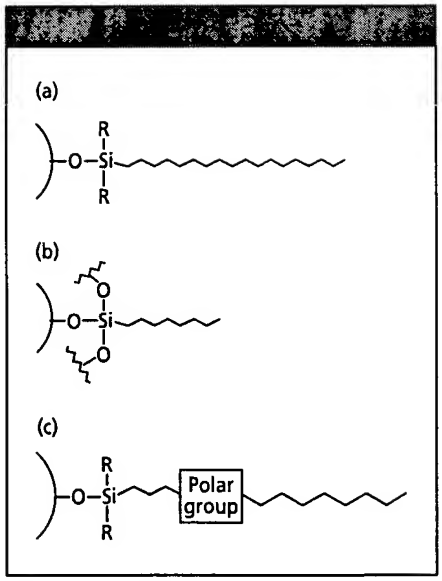

Figure 1: General structures of (a) monofunctional C18, (b) trifunctional C8, and (c) embedded-polar-group bonded phases. R = alkyl.

basic analytes (3,4). Other embedded polar functional groups such as ureas and carbamates were found to be similarly effective, and all three types of carbonyl phases now are commercially available.

In this article, we will compare the chromatographic properties of embedded-carbamate-group bonded phases with those of their purely alkyl counterparts. We expect that related amide, urea, and other embedded polar phases will behave in a similar manner, as long as analysts differentiate between the one- and two-step synthesis approaches (see below). Nonetheless, the chromatographic features described in this article are derived from a limited data set (column chemistry and test conditions) and should not be construed as universal for all column packings that contain embedded polar groups. As with any stationary phase, the utility of an embedded-polar-group bonded phase will depend upon the specifics of the separation problem.

Experimental

HPLC separations: We separated maleic acid (Aldrich Chemical Co., Milwaukee, Wisconsin) and toluamide and chlorpheniramine (maleate salt) (both from Sigma-Aldrich, St. Louis, Missouri) on a 150 mm × 4.6 mm, 5-µm d_p SymmetryShield RP_8 reversed-phase HPLC column (Waters Corp, Milford, Massachusetts) and on a 150 mm × 4.6 mm, 5-µm d_p Supelcosil ABZ+Plus reversed-phase HPLC column (Supelco, Bellefonte, Pennsylvania) as respective examples of second-generation, one-step and first-generation, two-step synthesis embedded-polar-group bonded phases. The HPLC system comprised an Alliance 2690 XE separations module, a model 996 photodiode-array detector, and a Millennium^{32} version 2.15.01 data-management system (all from Waters) and a Neslab RTE-111 circulating water bath (Neslab Instruments, Inc., Portsmouth, New Hampshire) for column temperature control. The mobile phase was 80:20 (v/v) 50 mM monobasic potassium phosphate (adjusted to pH 3.0 with phosphoric acid) and acetonitrile. The flow rate was 1.0 mL/min, and the temperature was 23.4 °C. The detection wavelength was 230 nm.

We separated uracil, propranolol (hydrochloride salt), and amitriptyline (hydrochloride salt) (all from Aldrich) and naphthalene, acenaphthene, and butyl paraben (all from Sigma-Aldrich) on 150 mm × 3.9 mm, 5-µm d_p SymmetryShield RP_{18} and Symmetry C_{18} columns (Waters) as respective examples of embedded-polar-group and conventional bonded phases. The HPLC system was the same as described above. The mobile phase was 35:65 (v/v) 20 mM monobasic potassium phosphate (adjusted to pH 7.0 with dibasic potassium phosphate)–methanol. The flow rate was 1.0 mL/min, and the temperature was 23.4 °C. The detection wavelength was 254 nm.

We separated suprofen, ketoprofen, naproxen, tolmetin (sodium salt), fenoprofen (calcium salt), diclofenac (sodium salt), indomethacin, and ibuprofen (all from Sigma-Aldrich) on 150 mm × 4.6 mm, 5-µm d_p Symmetry C_{18} and SymmetryShield RP_{18} columns (Waters) as respective examples of conventional and embedded-polar-group bonded phases. The HPLC system comprised a model 626 LC separations module with column heater, a model 486 tunable absorbance detector, a model 715 UltraWisp autosampler, and a Millennium^{32} version 3.2 data-management system (all from Waters). The mobile phase was 35:65 (v/v) 0.1% acetic acid–methanol. The flow rate was 1.0 mL/min, and the temperature was 24 °C. The detection wavelength was 254 nm.

Adsorption thermodynamic study: We separated benzamide, aniline, *m*-toluidine, benzoic acid, salicylic acid, phenol, and benzyl alcohol (all from Aldrich); benzonitrile, *o*-toluamide, and benzophenone (all from Sigma-Aldrich); and acetophenone (J.T. Baker, Phillipsburg, New Jersey) on 150 mm × 3.9 mm, 5-µm d_p SymmetryShield RP_8 and Symmetry C_8 columns (Waters) as examples of embedded-polar-group and conventional C8 bonded phases, respectively. The HPLC system included an Alliance 2690 XE separations module, a model 996 photodiode-array detector, and a Millennium^{32} version 2.15.01 data-management system (Waters). The column temperature was controlled using a model 2690 XE column heater (Waters). The mobile phase was 80:20 (v/v) 20 mM monobasic potassium phosphate (adjusted to pH 7.0 with dibasic potassium phosphate)–methanol. The flow rate was 1.0 mL/min, and the temperature was 40 °C. The detection wavelength was 254 nm.

Retention factor stability study in highly aqueous mobile phases: We separated sulfanilamide (Sigma-Aldrich) on 150 mm × 3.9 mm, 5-µm d_p SymmetryShield RP_{18}, SymmetryShield RP_8, Symmetry C_{18}, and Symmetry C_8 columns (Waters) as examples of embedded-polar-group and conventional bonded phases. The HPLC system comprised a model 600E solvent-delivery system, a model 490E programmable multiwavelength detector, a model 715 UltraWisp autosampler, and an ExpertEase data-management system (all from Waters). Column temperature was controlled using a Euramark Mistral column thermostat (Spark Holland, Mt. Prospect, Illinois). The mobile phases were 20 mM dibasic potassium phosphate (pH 6.0); 5:95 (v/v) methanol–20 mM dibasic potassium phosphate (pH 6.0); and 10:90 (v/v) methanol–20 mM dibasic potassium phosphate (pH 6.0). The flow rate was 1.0 mL/min, and the temperature was 25 °C. The detection wavelength was 254 nm.

Protocol: We equilibrated the columns for 30 min in methanol and then in 50:50 (v/v) methanol–water. Later, we equilibrated the columns for 30 min in 0–10% organic mobile phase and obtained the sulfanilamide retention factor (k) before stopping the flow. We stopped the flow for 1 h, and later resumed the flow in 0–10% organic mobile phase. We obtained the sulfanilamide retention factor after stopping the flow. The percentage decrease in retention factor was calculated using the following equation:

$$\text{Decrease in } k = 100\left(\frac{k_{\text{before}} - k_{\text{after}}}{k_{\text{before}}}\right) \quad [1]$$

Results and Discussion

Chromatographic differentiation of first- and second-generation phases: The first reported embedded-polar-group bonded phases were prepared by a two-step surface modification. In the first step, the native silica was bonded with an aminopropyl silane. In the second step, the amine groups were reacted with an acid chloride or isocyanate to form an amide or urea (2–5). This first-generation methodology suffers from less-than-complete conversion of the amine groups in the second surface reaction. As Figure 2 shows, the resulting surface can have a mixture of derivatized and underivatized amine groups. These first-generation phases have the potential to retain analytes by both reversed-phase and anion-exchange mechanisms (6–8).

The second-generation materials were prepared by a one-step surface modification in which the functional group was built into the silane (9). A single-surface reaction with the silane yields only one possible ligand structure with no possibility of anion-exchange functionality. O'Gara and coworkers (10) showed that this approach provided excellent batch-to-batch reproducibility for a set of polar, basic, and nonpolar analytes.

The absence of anion-exchange groups is an important advance with the second-generation materials. Figure 3 compares a

first- and a second-generation phase using a pH 3 buffered mobile phase for the separation of maleic acid, toluamide, and the base chlorpheniramine. Under these conditions, the analytes were negatively charged, neutral, and positively charged, respectively. For the one-step phase, all three analytes were eluted with short retention times and good peak shape. For the two-step material, the neutral and positively charged analytes were eluted in a similar fashion, but negatively charged maleic acid was strongly retained with poor peak shape. We attributed the strong retention of maleic acid to ion-exchange retention caused by residual amine groups on the surface. Thus, we recommend one-step materials over the older two-step materials for separations of negatively charged analytes.

Improved peak shape for basic compounds: The most sensitive measurement of silanol interactions is achieved using highly basic probes with a pH 7 mobile phase (11). At this pH, many of the residual silanols are in their ionized form ($Si–O^-$), and the basic probes are completely protonated (BH^+). The protonated bases interact with the ionized silanols by an ion-exchange mechanism, and the degree of tailing is a direct measure of silanol activity.

As Figure 4 illustrates, the *U.S. Pharmacopeia* (*USP*) tailing factor for amitriptyline was reduced almost 40% by substituting an embedded-polar-group C18 bonded phase for a conventional C18 bonded phase on silica (12). This carbamate-bonded-phase substitution reduced silanol interactions with basic analytes on hybrid organic–inorganic particles as well, in the form of a 15% decrease for amitriptyline's tailing factor. The diminished impact on the hybrid particles can be attributed to the inherently lower silanol concentrations generated by the methyl–silicon groups on the surface (13). O'Gara and colleagues (14) recently reported additional evidence to support reduced silanol interactions for embedded-polar-group bonded phases in an experiment that measured the tailing factors of two strong bases — propranolol and amitriptyline (both pK_as > 9) — as a function of ligand surface concentration. They found that the carbamate group reduced the interaction between bases and surface silanols in a pH 7 mobile phase, as indicated by a weak dependence of tailing factors on surface concentration. They observed a much stronger dependence for a conventional C18 bonded phase.

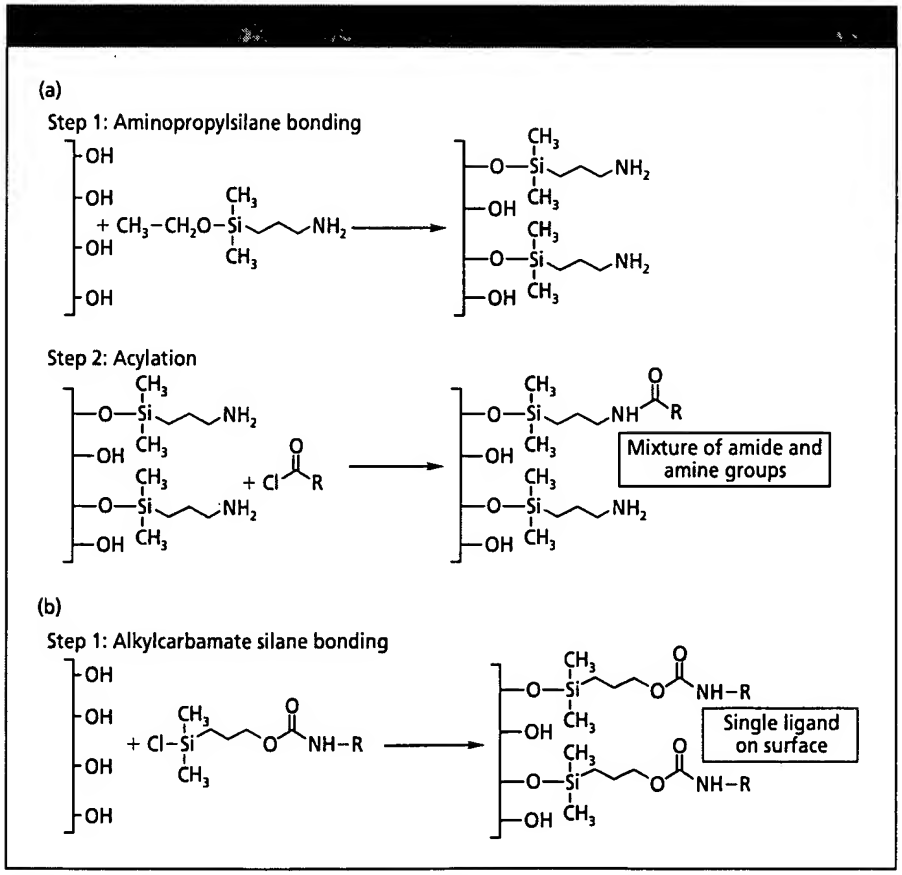

Figure 2: Examples of (a) first- and (b) second-generation approaches to the synthesis of embedded-polar-group bonded phases. R = alkyl.

Selectivity: Stationary phases bonded with embedded polar groups exhibit both retention and selectivity differences compared with their alkyl counterparts. Polar and basic analyte retention factors generally are reduced on embedded-polar-group phases, whereas nonpolar analytes are relatively unaffected (10). This effect is exemplified in Figure 4 between the nonpolar analyte acenaphthene and the base amitriptyline. Figure 5 illustrates a more complex selectivity change for the separation of a set of carboxylic-acid-substituted pharmaceuticals. The elution order changes for naproxen and talmetin and leads to better resolution on the embedded-polar-group bonded phase. Coeluted peaks for diclofenac and indomethacin were resolved by changing from the conventional C18 to the embedded-polar-group C18 column. Ibuprofen is eluted before diclofenac and indomethacin on the embedded phase.

We have used adsorption thermodynamic studies to compare retention mechanisms on alkyl and embedded-polar-group phases (15). As Figure 6 shows, the log of the retention factor (k) for a series of substituted benzenes was plotted comparing C8 alkyl with C8 carbamate phases (16). The slope of the plot is close to unity. Those eluites that fall on the line indicate similar retention energetics on both phases; however, a few eluites deviate from the regression line, which indicates the energetics behind selectivity are different. Anything above the regression line indicates that the energetics of retention are greater on the alkylcarbamate phase; anything below the line indicates that retention selectivity is greater on the alkyl phase. The three compounds that are more strongly retained on the C8 carbamate phase are the hydrogen bond donors benzamide, aniline, and phenol. Note that addition of a methyl substituent lowers the corresponding compound's location; for example, benzamide → toluamide, aniline → toluidine, and benzophenone → acetophenone. Benzoic acid falls below the line, but salicylic acid, with the additional hydroxy group, exhibits increased relative retention on the embedded-polar-group phase.

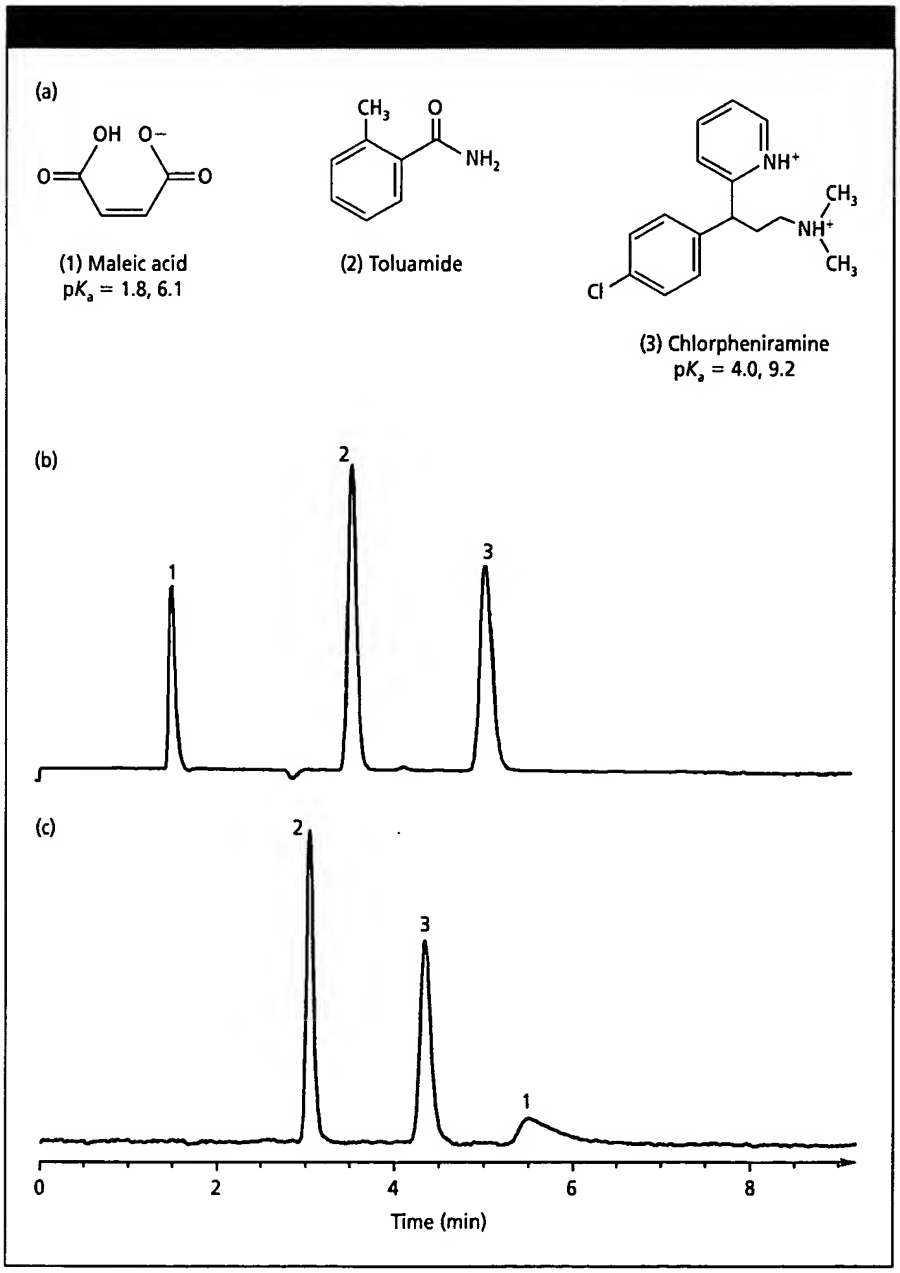

Figure 3: Separation of an acid, neutral, and base mixture at low pH using embedded-polar-group bonded phases prepared using one- and two-step synthesis methods. Shown are (a) structures of the analytes and chromatograms obtained using the (b) one- and (c) two-step phases.

Thus, selectivity differences between alkyl and embedded-polar-group bonded phases can be useful in method development. In many cases, when peaks are resolved unsatisfactorily on an alkyl phase, the embedded-polar-group counterpart might be substituted to achieve the desired separation with no mobile-phase adjustments.

Why embedded-polar-group bonded phases have different chromatographic properties than their alkyl counterparts remains the subject of ongoing investigation. Hydrogen bond donors may interact through hydrogen bonding with the embedded polar groups, which would explain the increased retention of this class of compounds on embedded-polar-group phases compared with conventional phases (17). Accounting for the reduced retention and tailing of basic compounds on embedded-polar phases is more difficult. Buszewski and colleagues (18) showed that the presence of an embedded polar group does not reduce the acidity of residual silanols. An alternative explanation is that the presence of the embedded polar group may affect the composition of the mobile phase adsorbed in the surface layer. For conventional alkyl-bonded phases, the surface layer contains a higher concentration of the organic component than the bulk mobile phase (18). Felitsyn and Cantwell (19) recently reported data showing that when organic modifier is sorbed into a C18 bonded phase, the sorbent properties are altered considerably. The surface layer of an embedded-polar-group bonded phase should have a higher concentration of the aqueous component because of the embedded functional group's hydrogen bonding ability. Jaroniec (20) demonstrated this effect for amide bonded phases. The embedded-polar-group bonded phase sur-

face layer should have a higher dielectric constant. As a result, the strength of ionic interactions between surface silanols and polar or basic compounds should be reduced and result in different selectivity and reduced tailing factors compared with the corresponding pure-alkyl-bonded phase (14). We expect a better mechanistic understanding of these novel phases with additional investigations.

Stable retention in highly aqueous mobile phases: Purely alkyl bonded phases can suffer to varying degrees from retention-time instability in 100% aqueous mobile phases (21). One cause of this instability is the poor wettability of alkyl surfaces by water (22,23). When the contact angle between the surface and the mobile phase is greater than 90°, pressure must be applied to force the mobile phase into pores that contain most of the surface area (24,25). Several factors can contribute to this wettability problem, depending upon the particular column packing material. Factors can include the pore size of the base silica, the bonding density, and the type of ligand on the particle's surface (26). For example, many of the newer alkyl C18 and C8 chromatographic packings have high bonding densities designed to improve peak shape for basic compounds and stability at high pH. However, the improvement in bonding density often can lead to retention-time instability in 100% aqueous mobile phases because of the highly hydrophobic nature of these phases. Purely alkyl phases with low bonding densities are less hydrophobic and can provide good retention-time stability in 100% aqueous mobile phases, albeit often with poor peak shape for bases.

Embedded-polar-group bonded phases can provide a solution to this dilemma. Recent reports have shown that these phases provide stable and reproducible analyte retention times in 100% aqueous mobile phases (26,27). Embedded-polar-group bonded phases may wet more easily for various reasons; certainly the hydrogen-bonding ability of an embedded functional group with water could drop the contact angle between the surface and water to less than 90°, at which water could penetrate the porous surface freely (22,23).

To probe the difference between alkyl and embedded-polar-group bonded phases, Walter and co-workers (26) measured retention factor stability in 90, 95, and 100% aqueous mobile phase using a stop flow test. In this test, when the flow was stopped and the pressure was released from the column, they found that the mobile phase extruded from the pores of the purely alkyl phases because of their poor wettability by the mostly aqueous mobile phases. After restarting the flow, they observed that the mobile phase remained outside of the pores, and the particle's accessible surface area was greatly diminished. As a result, the retention factors decreased. As Figure 7 shows, the percentage decrease in retention factor for sulfanilamide approached 100% for the alkyl phases in changing from 90:10 (v/v) water–methanol to 100% water. Embedded-polar-group phases were affected little across the three mobile phases — most likely because the aqueous mobile phase stayed within the pores, held by hydrogen bonding even without pressure on the column.

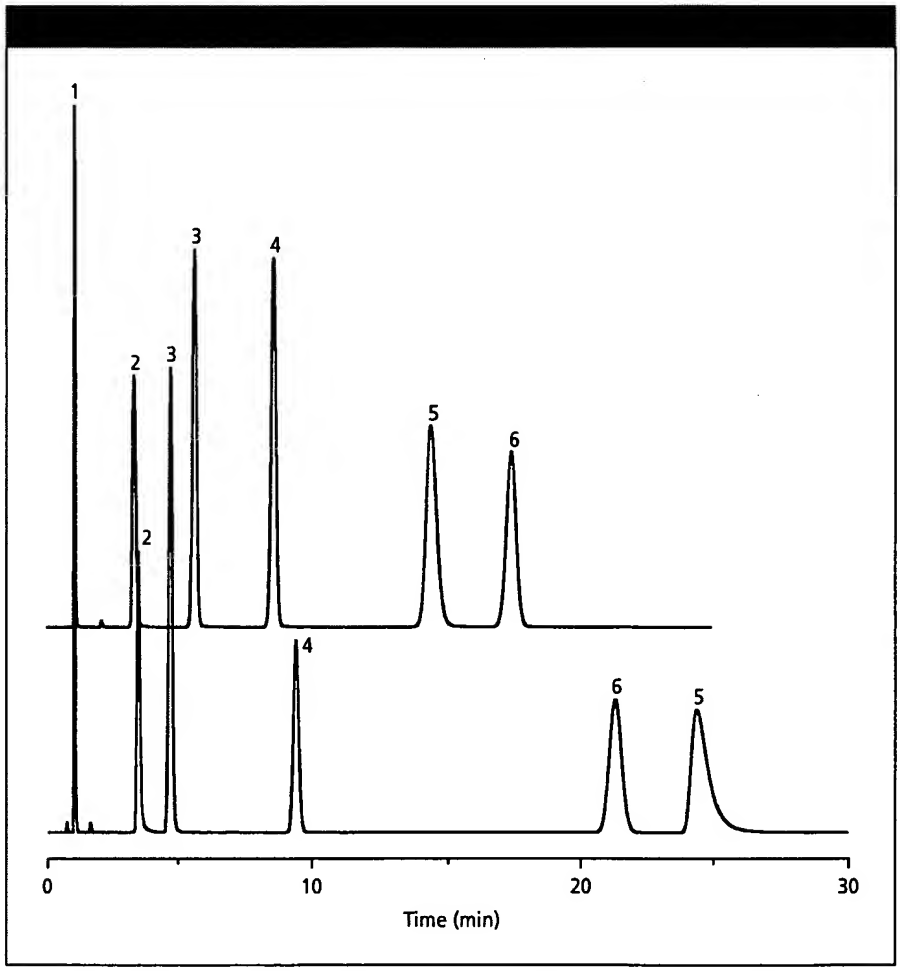

Figure 4: Chromatographic separation of polar, basic, and nonpolar analytes using an embedded-polar-group C18 phase (upper chromatogram, USP tailing factor = 1.1 for amitriptyline) and a conventional C18 stationary phase (lower chromatogram, USP tailing factor = 1.9 for amitriptyline). Peaks: 1 = uracil, 2 = propranolol, 3 = butylparaben, 4 = naphthalene, 5 = amitriptyline, 6 = acenaphthene.

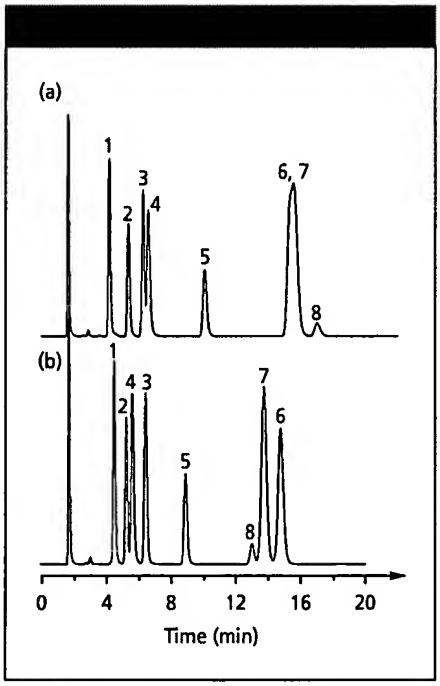

Figure 5: Chromatographic separation of acidic analytes using (a) conventional C18 and (b) embedded-polar-group C18 stationary phases. Peaks: 1 = suprofen, 2 = ketoprofen, 3 = naproxen, 4 = tolmetin, 5 = fenoprofen, 6 = diclofenac, 7 = indomethacin, 8 = ibuprofen.

Conclusion

Embedded-polar-group bonded phases for HPLC were introduced during the past 10 years. These phases represent a major departure from traditional silane and bonding technology. In comparison to their purely alkyl counterparts, embedded-polar-group phases may provide utility in three areas: improved peak shape for basic analytes, different selectivity, and stable retention in highly aqueous mobile phases. Researchers have made progress in understanding the mechanism of this class of reversed-phase materials. We expect more insight as these materials are used increasingly to solve difficult separation problems.

Acknowledgments

We thank M. Dion, R. Brennick, and M. Savaria of Waters Corp. for column packing. We would like to specially acknowledge U.D. Neue of Waters Corp., J.S. Petersen of GelTex Pharmaceuticals (Waltham, Massachusetts), and C.L. Niederländer of Sandoz AQ (Basel, Switzerland) for their pioneering work in the area of embedded-polar-group bonded phases.

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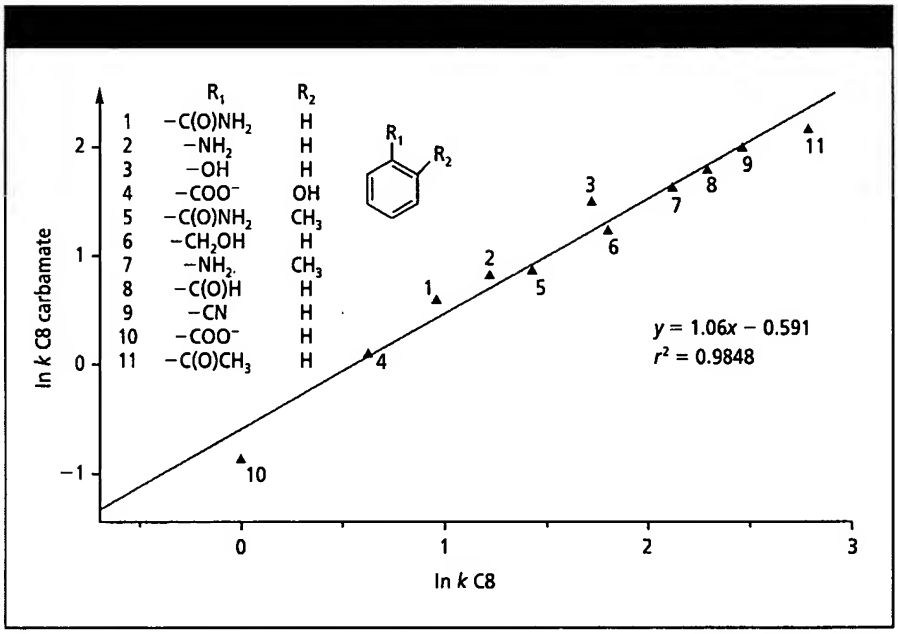

Figure 6: Log *k*/log *k* plot for substituted benzenes using embedded-polar-group C8 and conventional C8 stationary phases.

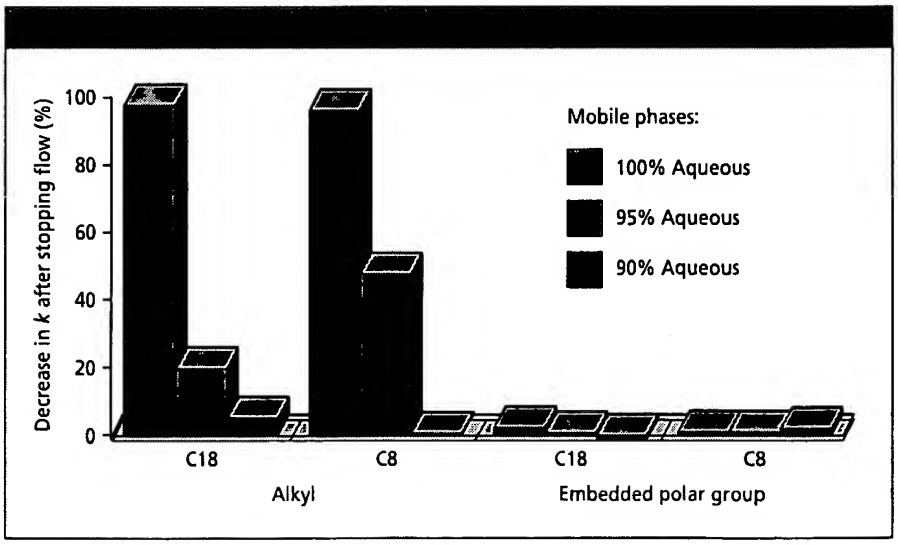

Figure 7: Comparison of the decrease in retention factor for sulfanilamide under stop-flow conditions on conventional alkyl and embedded-polar-group bonded phases.

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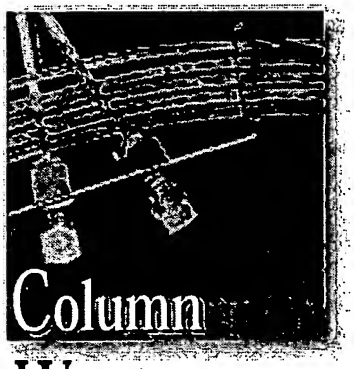

Watch

Phase Collapse in Reversed-Phase Liquid Chromatography

Matthew Przybyciel and Ronald E. Majors

This month's "Column Watch" examines the topic of phase collapse in reversed-phase liquid chromatography. Phase collapse is best described as a dewetting phenomenon. It can cause a total loss of retention and chromatographic problems such as peak tailing, nonreproducible retention times, and gradient regeneration delays. The authors offer suggestions for avoiding phase collapses and regenerating collapsed phases.

Ronald E. Majors
Column Watch Editor

Reversed-phase liquid chromatography (LC) is the most popular mode of high performance liquid chromatography (HPLC) (1). Its continued popularity no doubt exists because of its versatility, its familiarity to most liquid chromatographers, the number and variety of commercial phases available, and the large number of published applications. Reversed-phase chromatography can be used to separate nonpolar, polar, and ionic compounds — sometimes in the same separation. The technique also can achieve a larger range of separations than all other modes combined because it enables users to manipulate the mobile phase by changing organic solvent type, solvent composition, and pH; by adding modifiers such as surfactants, chiral reagents, competing bases, and ion-pair reagents; or by adjusting experimental conditions such as flow rate and temperature.

Reversed-phase chromatography generally is performed using octadecyldimethylsilane (C18) or octyldimethylsilane (C8) stationary phases bonded to high-purity, spherical silica gel. Short-chain alkyl phases such as C2 and C4 and phenyl phases are used occasionally, but long-chain phases such as C30 are used rarely and only for specialized applications. Polymeric materials such as poly(styrene–divinylbenzene) (PS–DVB) find some use as reversed-phase media (for example, with high-pH mobile phases) but generally provide lower column efficiencies than those of silica gel–based packings. Water, usually buffered, mixed with a water-miscible organic solvent (modifier), most commonly acetonitrile or methanol, is the preferred mobile phase. To retain most nonpolar and semipolar organic analytes, chromatographers generally begin method development by varying the water–organic solvent ratio in the 20–80% range, often using gradient elution to find the optimum separation conditions more quickly.

Classic Explanation of Phase Collapse with Highly Aqueous Mobile Phases

With this type of approach, chromatographers can achieve a wide variety of separations. However, even the lower end of the 20–80% concentration range could be too strong for adequate retention for very polar analytes such as small organic acids or purine bases. For solubility reasons, many polar compounds separate better with an aqueous mobile phase and can be retained only with a minimal concentration, sometimes less than 5%, of organic modifier. Because the stationary phase in reversed-phase chromatography usually is quite hydrophobic — especially when a densely bonded, long-alkyl-chain phase is used in a highly aqueous and polar mobile phase — these oil-like phases tend to minimize their surface energy through an increased disposition to self-associate. The surfaces can dewet in this process, which occurs within the porous structure of the packing material. When this phenomenon occurs, the polar analytes can have trouble partitioning into or adsorbing onto the stationary-phase surface due to the poor wettability by water. From a chromatographic viewpoint, the stationary phase is believed to have collapsed. Others call this occurrence chain folding or stationary-phase matting. Figure 1 shows a rather simplistic view of this phenomenon. Of course, because most of the bonded phase is inside the pores of the silica gel, this collapse would occur within them rather than on the outer surface, as Figure 1 depicts.

The overall result of a phase collapse is that chromatography becomes more problematic with retention loss, retention irreproducibility, increased tailing, and long gradient regeneration times (2). Many variables affect the rate and degree of retention loss, including the type of bonded alkyl ligand, the bonding density, and the pore diameter of the silica. Phase collapse is less of an issue when the stationary-phase cover-

age (bonding density) is low (less than 3 μmol/m^2), presumably because the silanols that remain on the surface allow the surface to be wetted with water molecules and allow the alkyl chains to continue to interact with the hydrophobic portion of the polar analyte. Phase collapse also is rare when very short, alkyl-bonded phases such as C3 are used, because the available alkyl surface is determined by the high carbon density of compact bonded phase, little free volume exists between bonded chains, and little shielding of the silanols occurs. Although chromatographers have speculated about what is happening mechanistically, the phenomenon has not been studied systematically.

Wetting Phenomenon for Alkyl Phases

Kazakevich, LoBrutto, and co-workers (3,4) have studied the phenomenon from practical and theoretical viewpoints by investigating bonded alkyldimethylsilanes of different chain lengths from C1 to C18 on the same silica. They looked at the molecular volumes of bonded alkyl ligands, the decrease in pore volume with increasing chain length and bonded-phase coverage, and the measurements of the bonding density of

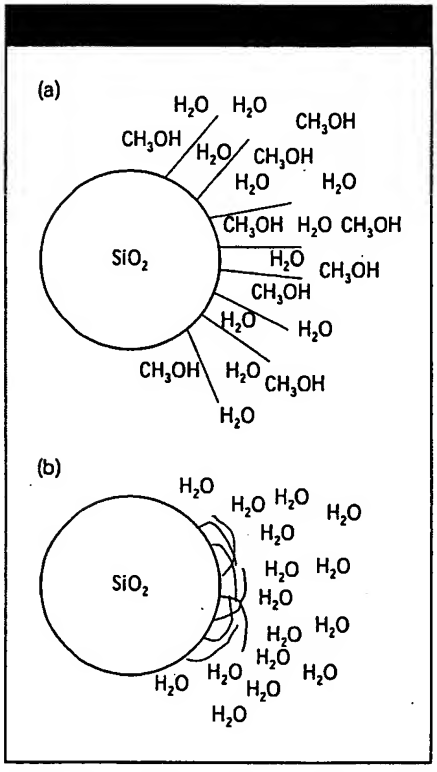

Figure 1: Illustration of the classic explanation of phase collapse in reversed-phase chromatography. Shown are the configurations of long-chain bonded alkyl phases (a) in water–methanol mixtures and (b) in 100% water.

different phases in the presence of typical HPLC mobile phases. Based on their observations, they concluded that alkyl phases always are in the most compact conformation (that is, a collapsed state) regardless of the concentration of organic modifier. They found that the accumulated amount of eluent component in the stationary phase practically was independent of bonded alkyl-chain length. In addition, they observed that when flowing 100% aqueous mobile phase overnight through an alkyl phase column at a low flow rate, the elution volume of the void marker was lower than a predetermined void volume. They attributed this behavior to the inability of the 100% aqueous mobile phase to penetrate the silica pores because of water's high surface tension — a phase wetting problem. However, by resolvating the phase (flowing a high concentration of acetonitrile for at least 3 h), the elution volume of the void marker was restored.

This wetting phenomenon is very similar to the one described by Bouvier and colleagues (5) in their development of a novel reversed-phase sorbent for solid-phase extraction (SPE). A water-miscible organic solvent normally is used to wet a silica-based, reversed-phase SPE sorbent in a conditioning step before its use to extract an aqueous sample. If no conditioning occurred, then the hydrophobic sorbent inside the pores would not wet with water. After a sorbent is wetted, water or aqueous buffer can displace the filled pores. A dry sorbent requires very high pressure to cause aqueous solvent to enter the pores, according to the Laplace–Young equation (equation 1), which relates the intrusion pressure to the surface tension of the water and to the contact angle of the water and air in the sorbent surface (6):

$$\Delta P = \frac{4\gamma \cos\theta}{d} \qquad [1]$$

where ΔP is the intrusion pressure required to drive liquid into the pores, γ is the surface tension, d is the effective pore diameter, and θ is the contact angle made between water and air on the adsorbent surface. The actual measurement of contact angle is difficult with a nonuniform porous surface such as a bonded silica gel, but it has been approximated by Bouvier and co-workers (5) by considering a water–paraffin–air system.

To chromatographically illustrate the wetting–dewetting phenomenon and phase collapse, Przybyciel and Santangelo (7) used amoxicillin, a polar antibiotic, as a test probe. Figure 2 shows a series of chromatograms obtained using the same classic C8 endcapped column. The column was treated first with a water–acetonitrile mobile phase until they observed a stable baseline. Then, they switched the mobile phase to 0.1% acetic acid in water (no organic solvent present). The initial chromatogram (Figure 2a) showed an amoxicillin retention time of 8.6 min. Next, they shut off the pump and allowed the system to rest for 10 min. They resumed the flow with the same mobile phase and injected another amoxicillin sample; Figure 2b is the resulting chromatogram. Note the retention time of 3.5 min for amoxicillin. The reduced retention time and poor peak shape can be attributed to the exclusion of water–acetonitrile from the pores with resultant dewetting (desolvation) of the bonded phase and subsequent collapse.

Restoring a Column to its Original State

A column can be restored to its original state by repressurization or resolvation. The Laplace–Young equation describes the pressure needed to drive water completely into the pore structure (8). The contact angle with a 100% aqueous mobile phase and an alkyl-bonded phase in a silica-gel pore is greater than 90°; therefore, the surface is not wetted. Pressure must be applied to a column to cause water to be forced back into the pores.

To restore the column retention for amoxicillin, Przybyciel and Santangelo (7) performed an experiment in which they pressurized a column to 270 bar and used a restrictor and the same 0.1% acetic acid in water as the mobile phase. The column was allowed to pressurize under flow conditions for 10 min, after which time the restrictor was removed but the original column flow was maintained. The amoxicillin sample was reinjected into the column. The chromatogram of amoxicillin (Figure 2c) now shows an increased retention time (7.8 min) for amoxicillin. They believed that pressurizing the column was sufficient to drive water into the silica pores and enable the interaction with the alkyl chain. The amoxicillin was not restored completely to its original retention time because the pressure needed to be greater.

They shut off the pump again and released the pressure. As expected, the retention time for amoxicillin was reduced again as the aqueous mobile phase was extruded from the pores. After restarting

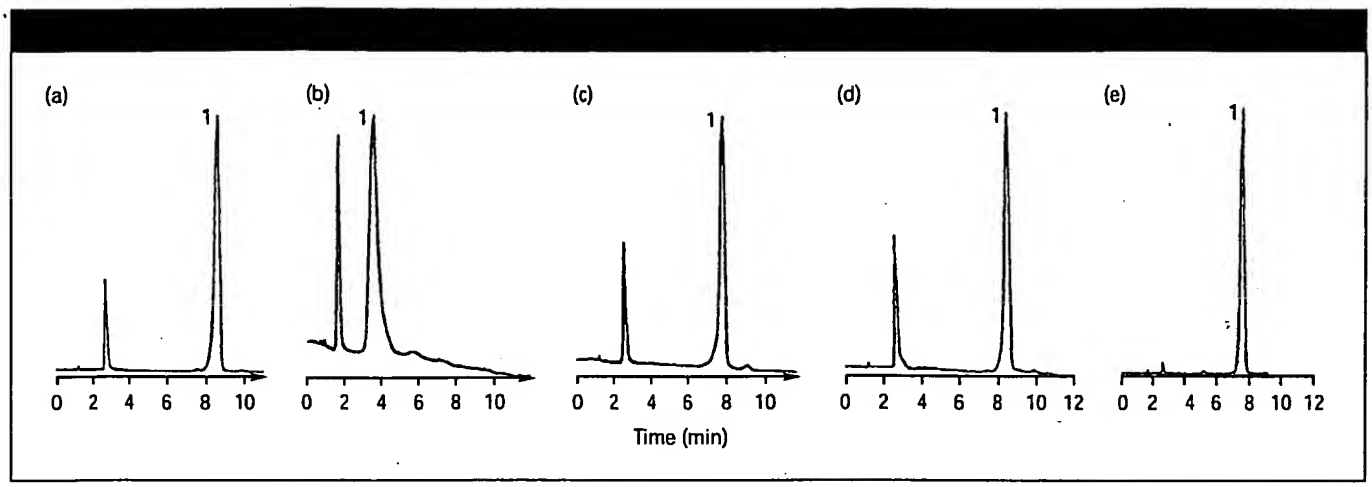

Figure 2: Chromatograms showing phase collapse in reversed-phase chromatography with highly aqueous mobile phase (7). Mobile phase: 0.1% acetic acid in water; flow rate: 1.0 mL/min; sample: amoxicillin dissolved in water. Shown are results from injection of an amoxicillin sample (a) onto a reversed-phase column that had been conditioned with a water–acetonitrile mobile phase (40:60 v/v), (b) after the pump was shut off for 10 min and flow was resumed, (c) after the column was pressurized to 270 bar with a restrictor and continuous flow, (d) after the column was conditioned with water–acetonitrile (40:60 volume %), and (e) onto a column with a polar-embedded bonded phase (alkyl phase containing ether linkage).

the flow, an aqueous mobile phase no longer entered the pores, and the accessible surface area was diminished greatly. Next, they exposed the column to a 60:40 (v/v) acetonitrile–water mobile phase for 30 min at 1 mL/min. They switched the column back to the 0.1% acetic acid in water mobile phase and injected amoxicillin. Figure 2d shows a chromatogram from this analysis. Note the restored retention time for amoxicillin. The water–organic solvent mobile phase easily wetted the C8 phase and occupied the pores of the silica. These pores contained mobile phase with a high content of acetonitrile, which sufficiently lowered the surface tension of the phase and allowed water to penetrate the silica pores at the column's normal operating pressures. However, if the 0.1% acetic acid mixture were allowed to flow through the restored column for a long period of time, the retention time for amoxicillin would be reduced because the acetonitrile in the pores would be removed slowly, and the water once again would be excluded from the pores. Other researchers have observed similar dewetting phenomena of alkyl-bonded phases (2,8,9).

To overcome this wetting–dewetting phenomenon, Przybyciel and Santangelo (7) used a stationary phase engineered for use with high-water-concentration mobile phases. They obtained the chromatogram in Figure 2e with a 7.5-min retention time and a good peak shape for amoxicillin. Using this type of column, they achieved a stable retention time for amoxicillin under all operating conditions and independent of the column pressure. This type of column is designed to allow water to penetrate the pores at normal operating pressures using all mobile-phase compositions, including 100% water.

Figure 3 is a simple illustration of this proposed dewetting phenomenon (8,10). If we start with a mobile phase of water and a water-miscible organic solvent such as acetonitrile so that the pore is wetted, bonded hydrocarbon chains are solvated, extended, and ready to interact with the polar analyte. Normal retention occurs. With pressure and 100% water flowing through the pores because of the pressure, the bonded phase is in an extended state and can interact with analytes, and the analytes are properly retained inside the pore (Figure 3a). However, if we stop the flow and the pressure that was forcing aqueous mobile phase into the pore ceases, the hydrophobic pore surface can expel the polar mobile phase and dewet the pore (Figure 3b). Analytes no longer can enter the pore and interact with the bonded phase, and, even if the flow is restarted, the pores are still dewetted and the analytes are unretained. If an organic solvent such as methanol or acetonitrile or a water mixture with a substantial amount of organic solvent is pumped through the column, the pores can become rewetted and normal retention can recur.

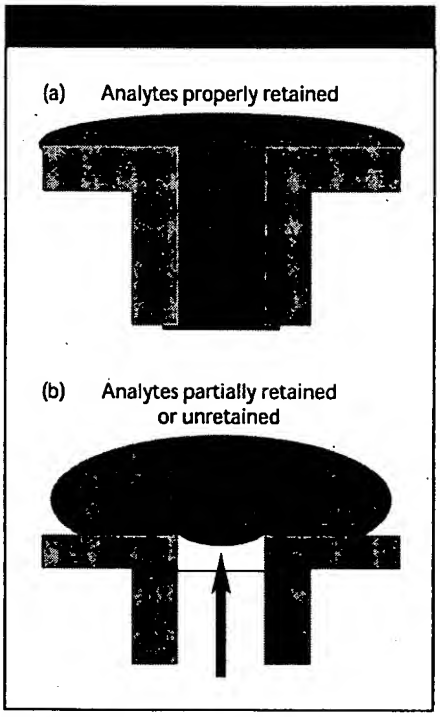

Figure 3: Illustration of a possible mechanism of pore dewetting for reversed-phase chromatography in a highly aqueous mobile phase (8) in which (a) the alkyl chains are properly solvated with pressure using a 100% aqueous mobile phase and (b) the flow has been stopped to allow expulsion of water from the pores; with flow resumed the pores are still dewetted and analytes cannot enter pores and have little or no retention.

Approaches for Successful Reversed-Phase Chromatography in Highly Aqueous Mobile Phases

Several approaches in designing stationary phases can help retain polar analytes under highly aqueous conditions, including using

- nonendcapped short-chain alkyl phases;
- hydrophilic, polar-endcapped, and polar-enhanced stationary phases;
- polar-embedded alkyl phases;

- long-chain alkyl phases; and
- wide-pore diameter phases.

In next month's "Column Watch" column, we will discuss these approaches in detail and provide several examples of the successful use of reversed-phase columns in highly aqueous mobile phases — even 100% water — with excellent peak shape, good chromatographic retention, and rapid gradient reequilibration.

Conclusions

In this month's "Column Watch," we described and demonstrated the phenomenon of phase collapse when using alkyl bonded phases (such as C8 or C18) in reversed-phase LC with water or mobile phases that have a low percentage of organic solvent. Because of surface tension, organic solvent is expelled from the pore unless the pressure is high enough to keep the phase in the pore solvated or the porous structure has a sufficient concentration of organic modifier to keep the phase solvated. Phase collapse is best described as a dewetting phenomenon. In its extreme, phase collapse (dewetting) can cause a total loss of retention. In normal practice, it can occur with organic solvent concentrations of less than 5% in the aqueous mobile phase and retention can be reduced gradually. Where and when it occurs depends upon the nature of the bonded phase, the density of the bonded phase, and the pore diameter of the packing. Phase dewetting causes chromatographic problems such as retention loss, peak tailing, nonreproducible retention times, and gradient regeneration delays. Instrument problems such as unreliable solvent compositions at low percentages of organic solvent in a binary gradient pumping system also can cause nonreproducible retention.

Phase collapse does not damage a column, and washing a column with a 50% or higher concentration of the organic solvent can regenerate the column. However, after a period of time using a low percentage of organic solvent, phase collapse can recur. Phase collapse can be avoided by using columns especially designed for operation in highly aqueous environments. Many of these columns also can be used as regular reversed-phase columns throughout the entire range of aqueous-to-organic solvent mobile phase. These columns will be described in detail in next month's "Column Watch."

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